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# Application of NMR Methods to Identify Detection Reagents for Use in the Development of Robust Nanosensors

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## Abstract

Nuclear Magnetic Resonance (NMR) spectroscopy is a powerful technique for studying bi-molecular interactions at the atomic scale. Our NMR lab is involved in the identification of small molecules, or ligands that bind to target protein receptors, such as tetanus (TeNT) and botulinum (BoNT) neurotoxins, anthrax proteins and HLA-DR10 receptors on non-Hodgkin's lymphoma cancer cells. Once low affinity binders are identified, they can be linked together to produce multidentate synthetic high affinity ligands (SHALs) that have very high specificity for their target protein receptors. An important nanotechnology application for SHALs is their use in the development of robust chemical sensors or biochips for the detection of pathogen proteins in environmental samples or body fluids. Here, we describe a recently developed NMR competition assay based on transferred nuclear Overhauser effect spectroscopy (trNOESY) that enables the identification of sets of ligands that bind to the same site, or a different site, on the surface of TeNT fragment C (TetC) than a known 'marker' ligand, doxorubicin. Using this assay, we can identify the optimal pairs of ligands to be linked together for creating detection reagents, as well as estimate the relative binding constants for ligands competing for the same site.

## Key words:

Nuclear Magnetic Resonance, structure-based drug design, transferred nuclear Overhauser effect spectroscopy, tetanus toxin, biosensors

## 1. Introduction

Nuclear Magnetic Resonance (NMR) spectroscopy has evolved into an important technique in support of structure-based drug design because of its long tradition in the study of molecular interactions [1, 2]. Several NMR experiments have been used in generic binding assays to identify weak, but specific, binding between small molecules and a target protein. The advantage of these NMR screening methods is that they can be applied as soon as a target protein is available without the need for extensive assay development. NMR screening methods have been reviewed extensively by others [3-5] and will not be described in detail here, with the exception of the transferred nuclear Overhauser effect spectroscopy experiment (trNOESY).

TrNOESY experiments [6-8] are routinely used to detect ligand binding to a target protein under conditions of fast exchange (ligands that bind with μM to mM dissociation constants). The advantages of the trNOESY method are that it does not require large amounts of pure, labeled protein, it is not limited by the size of the protein, and it can provide information about the structure of bound form of the ligand. In the experiment, the intensity of each intra-ligand NOE crosspeak is governed by the population-weighted cross-relaxation rate [9] (Figure 1). Thus the binding event is relatively straightforward to detect and does not require time-consuming chemical shift assignments. A strong negative NOE crosspeak is observed for binders, as opposed to weakly positive or zero NOE crosspeaks for the same compounds in the absence of the target receptor, as shown for MP-biocytin in Figure 2. Thus the sign flip of the NOE cross peak between the free versus bound states acts as a simple binary filter to distinguish binders from nonbinders [10, 11] (*see* **Note 1**).

An important parameter in selecting a reagent for use in a sensor is its relative binding affinity. Very recently, several labs, including our own, have begun to address the issue of whether NMR screening methods can simultaneously and rapidly provide this information [12-14]. For the preparation of multivalent ligands that consist of two or more ligands that are weak binders, it is also useful to determine if each ligand is binding with specificity to the same site or to a different site than another ligand. Although the individual ligands that comprise a linked bidendate compound may only bind weakly to the protein, as expected because of their small size, the free energy of binding of the linked compound is, in principle, the sum of the free energies of each fragment plus a term due to linking [15]. Thus, linked compounds with < μM dissociation constants can be obtained by linking two fragments that each dissociate in the > μM range [16]. Here, we present the methods in detail, for carrying out a trNOESY competition binding assay for tetanus toxin fragment C (TetC).

Tetanus toxin (TeNT) and the botulinum toxins (BoNTs) are structurally and functionally related members of the family of *Clostridial* neurotoxins. The recent interest in these neurotoxins arises from the increased frequency of BoNT's use in medicine, occasional diary cattle and wildfowl deaths that have resulted from toxin ingestion and the potential threat that this protein might be used by terrorist groups or other nations as a biological weapon [17, 18]. Both toxins selectively concentrate at the synapse of axons in vertebrate motor neurons and are the most potent toxins known to man [19]. The entry of these toxins into neuronal cells requires the initial binding of the toxin to gangliosides on the cell surface. Thus effective inhibitors that block neuronal cell binding can be

developed for use as antidotes or serve as molecular recognition materials for affinity-based chemical sensors that detect and identify these highly toxic proteins.

## 2. Materials

1. High field NMR instrument (≥ 500 MHz) (*see* **Note 2**)
2. Available Chemical Directory or similar library of compounds to screen
3. Recombinant tetanus toxin C fragment (TetC) (Roche Molecular Biochemicals, Indianapolis, IN)
4. 100% Deuterium oxide ($D_2O$) (Isotech Inc., Miamisburg, OH)
5. 100% Deuterated dimethyl sulfoxide (DMSO) (Isotech Inc., Miamisburg, OH)
6. Doxorubicin hydrochloride (Sigma-Aldrich Chemical Co., St. Louis, MO); *Caution*, the antitumor drug doxorubicin is an inhibitor of reverse transcriptase and RNA polymerase, is an immunosuppressive agent and it intercalates into DNA [20-22].
7. 3'-Sialyllactose (Sigma-Aldrich Chemical Co., St. Louis, MO)
8. Sarcosine-Arg-Gly-Asp-Ser-Pro (Sar-RGDSP) (Sigma-Aldrich Chemical Co., St. Louis, MO)
9. 3-(N-maleimidopropionyl)biocytin (MP-biocytin) (Sigma-Aldrich Chemical Co., St. Louis, MO)
10. Lavendustin A (Sigma-Aldrich Chemical Co., St. Louis, MO); *Caution*, lavendustin A is a protein tyrosine kinase inhibitor [23].
11. Naphtho-fluorescein-di-β-galactopyranoside (NF-GalPyr) (Sigma-Aldrich Chemical Co., St. Louis, MO).

12. Ser-Gln-Asn-Tyr-Pro-Ile-Val (SQNYPIV) (Calbiochem-Novabiochem Corp., La Jolla, CA)
13. Sialic acid ) (Calbiochem-Novabiochem Corp., La Jolla, CA)

## 3. Methods

The methods described below outline (1) the theory of the trNOE experiment, (2) the NMR experimental setup (3) NMR sample preparation, and (4) the combined screening/competition trNOESY assay.

### *3.1. Theory of the Transferred Nuclear Overhauser Effect Experiment*

At spectrometer frequencies of 500-600 MHz ($H_v$), small molecules (MW < 1500 Da) in the free-state yield small, positive NOEs, while large protein molecules (MW > 10,000 Da) yield large, negative NOEs. In the fast exchange regime (binding constant between $10^{-3}$ and $10^{-7}$ M), the ligand acquires the NOE characteristics of the large molecule during the reversible binding and shows large negative NOEs (Figure 1). These characteristics are transferred from the bound state of the ligand to its free state, and therefore the ligand signals are still sharp due to the rapid total rotational correlation time, $\tau_c$ of the free ligand. The ligand binding event is thus identified by both the change in sign and the intensity buildup rate of its intramolecular NOEs.

The theory behind the mechanism of trNOE has been well developed over the years [24-29]. The dynamics of the NOE is governed by the three species equilibrium process given by:

$$[P] + [L] \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} [PL], \qquad [1]$$

where, [P], [L] and [PL] are the molar concentrations of the protein, the ligand and the complex, respectively, and $k_{on}$ and $k_{off}$ are the association and dissociation constants, respectively. In the trNOE experiment, the exchange of the ligand between the free and bound states alters the relaxation dynamics of the ligand more significantly than of the protein. Under equilibrium conditions, the binding constant $K_D$ is the ratio of $k_{off}$ to $k_{on}$. The exchange rate that is relevant to the NMR experiments, $k_{ex}$ depends on the relative populations of the protein and ligand as well as the binding constant, and is defined as

$$k_{ex} = k_{on}[P] + k_{off} = k_{off}/(1-L_b), \qquad [2]$$

where, $L_b$ is the bound ligand fraction. For a single ligand binding site, $L_b$ is given by

$$L_b = [(P_T+L_T+K_D) - ( \sqrt{(P_T+L_T+K_D)^2 - 4P_TL_T} )]/2L_T, \qquad [3]$$

where, $P_T$ and $L_T$ are the total protein and ligand concentrations, respectively, and $K_D$ is the binding constant. In the performance of trNOE experiments, it is useful to have the protein binding site at least half-saturated ($L_T \approx K_D$). This is accomplished by using a large molar excess of the ligand of approximately 5 to 50 times that of the protein. Exchange of the magnetization between the protein bound form and the free form of the ligand produces an averaged NOE.

The exchange averaged NOE depends on the rate of exchange and the magnitude of the NOE between the free ($NOE_f$) and bound ($NOE_b$) forms. The exchange rate, $k_{ex}$, is considered fast, intermediate or slow if $k_{ex} \gg |NOE_f - NOE_b|$, $k_{ex} \approx |NOE_f - NOE_b|$ and $k_{ex} \ll |NOE_f - NOE_b|$, respectively. In the fast exchange regime ($k_{ex} \gg |NOE_f - NOE_b|$), the

trNOE experiments are extremely useful as the observed NOE is then a population weighted average.

The dynamic interplay between the ligand intramolecular NOEs and the ligand/protein intermolecular exchange NOEs can be described by combining Solomon's equations and chemical exchange equations [30-32]. Using this approach, Clore and Gronenborn described the observed effect of trNOE by using matrix notation [25, 26]. The combined matrix includes the pairwise interactions in a multiple-spin system undergoing exchange, which accounts for the spin diffusion effects as well [29, 33, 34]. The evolution of the intensity in a two dimensional trNOE experiment (trNOESY) is given by [30, 32]:

$$\frac{d}{dt_m}\mathbf{V}(t_m) = -\mathbf{\Gamma V}(t_m), \qquad [4]$$

where $\tau_m$ is the mixing time, and the elements of the matrix $\mathbf{V}(\tau_m)$ are the measured peak volumes of the crosspeaks in the trNOESY spectrum, which are described in terms of the exchange-relaxation matrix $\mathbf{G}$. The exchange rates ($k_{ex}$) as well as the self ($\rho_{ij}$) and cross relaxation ($\sigma_{ij}$) rates of the various proton pairs are included in $\mathbf{G}$. When the exchange is fast relative to the relaxation rates, the effective rate constants are molar fraction weighted averages of the rate constants of the free and bound forms. Thus, if 'i' and 'j' are the ligand spins, the effective cross relaxation rate $\sigma_{ij}^{avg}$ is

$$\sigma_{ij}^{avg} = L_b\,\sigma_{ij}^{b} + (1 - L_b)\,\sigma_{ij}^{f}. \qquad [5]$$

In this fast exchange limit, as pointed out by Landy and Rao [35], the relaxation + exchange matrix, $\Gamma$, can be symmetrized and thus the rate equation for the m-spin ligand and n-spin protein simplifies to a (n + m) differential equation [36]. The relative

concentrations of the ligand and protein, following Zabell and Post [37] and Eq.[2] can be written as:

$$\frac{d}{dt}_m \begin{pmatrix} \left(V_l^b + V_l^f\right) \Big/ \sqrt{m_p^b} \\ \left(V_p^b + V_p^f\right) \Big/ \sqrt{m_l^b} \end{pmatrix} = -\begin{pmatrix} m_l^b \Gamma_l^b + m_l^f \Gamma_l^f & \sqrt{\left(m_l^b m_p^b\right)}\Gamma_{lp}^b \\ \sqrt{\left(m_l^b m_p^b\right)}\Gamma_{lp}^b & m_p^b \Gamma_p^b + m_p^f \Gamma_p^f \end{pmatrix} \times \begin{pmatrix} \left(V_l^b + V_l^f\right) \Big/ \sqrt{m_p^b} \\ \left(V_p^b + V_p^f\right) \Big/ \sqrt{m_l^b} \end{pmatrix} \quad [6]$$

where, $\Gamma_l^b$ and $\Gamma_l^f$ represent the symmetrical n × n relaxation matrices of the ligand in the bound and free forms, respectively and $\Gamma_p^b$ and $\Gamma_p^f$ have analogous definitions for the protein. The relative concentrations are defined by 'μ' as

$$\mu^b_l = [PL]/([PL]+[L]),$$

$$\mu^f_l = [L]/([PL]+[L]),$$

$$\mu^b_p = [PL]/([PL]+[P]),$$

$$\mu^b_l = [P]/([PL]+[P]). \quad [7].$$

These equations are generally used in cases where quantification of ligand crosspeaks in the trNOESY spectra is needed to determine the structure of the bound form of the ligand. This information is especially useful when combined with computational approaches in order to optimize models of protein/ligand complexes, which in turn are used to develop new ligands with higher affinity and specificity for the target site on the protein.

### *3.2 NMR Experimental Setup*

This section describes: (1) NMR pulse sequences, which are composed of a series of radio frequency pulses interspersed with delays, and are the actual NMR experiments, (2) the selection of the mixing time for the trNOESY experiment, and (3) experiment time and effects of temperature on the trNOESY experiment.

#### *3.2.1. NMR pulse sequences*

The pulse sequence for the trNOESY experiment differs slightly from the conventional three-pulse NOESY experiment [38], as shown in Figure 3.

3.2.1.1. Filtering out protein signals (*see* **Note 3**).

One common modification is the insertion of a relaxation filter to suppress extraneous protein signals, which may interfere with the detection of the ligand signal. This can be achieved by the $T_{1\rho}$ (or $R_2$) filter. This filter could either be introduced prior to or after the first 90° pulse. If introduced prior to the first pulse, then the filter needs to be flanked by additional 90° pulses or by using windowless multiple pulse sequences such as DIPSI [39], so that the magnetization is along the Z-axis after the filter is employed. If the filter is introduced after the first 90° pulse, as in the original experiment proposed by Scherf and Anglister [40], care must be taken to acquire the first $t_1$ point to avoid the need for large phase corrections in the indirect dimension.

3.2.1.2. Suppression of the water signal (*see* **Note 4**).

Another common modification to the pulse sequence is the control or suppression of the water magnetization. There are a variety of pre-experimental water suppression experimental schemes available, such as excitation sculpting based suppression [41] or WET [42, 43]. Post-experimental water signal elimination using methods such as water-gate or its modifications [44, 45] are also a viable alternative to pre-elimination. When considering water suppression schemes in a trNOESY experiment, it is critical to consider how the water suppression will effect the detection of the ligand signals. For example, if the ligand contains exchangeable protons, then it is desirable to avoid presaturation as it tends to saturate these resonances as well. It is also important to note that all the post-experimental water suppression schemes (e.g. water-gate) have a non-uniform excitation profile near the water frequency. Under such conditions, it is advisable to adjust the sample temperature to move the water signal so that any nearby ligand resonances do not get suppressed as well.

*3.2.2. Selection of Mixing times*

For screening purposes, it is first useful to identify where the ligand protons resonate when protein is not present. This requires that a NOESY spectrum of the ligand be obtained. A direct comparison of the spectrum with ligand alone and the spectrum of the ligand or mixtures of ligands in the presence of the TetC protein provides a simple and rapid way of identifying those crosspeaks that belong to that particular ligand without having to assign the chemical shifts. Assignment of chemical shifts can often be a very

time-consuming process, and would only be needed if the structure of the bound form of the ligand is to be determined.

In the absence of protein, the NOESY experiments are carried out at longer (600 to 900 ms) mixing times, while mixtures of ligands in the presence of TetC are carried out at shorter (200 and 300 ms) mixing times. Long mixing times are necessary for detection of NOEs for small molecules (< 1 to 2 kDa MW) because the product of $\omega_o$ ($2\pi$ x spectrometer frequency) and $\tau_c$ (rotational correlation time) is less than 1 ($\omega_o\tau_c < 1$); in contrast, shorter mixing times are required for large molecules or ligands binding to large molecules because $\omega_o\tau_c >> 1$ [46].

*3.2.3. Experiment time and Temperature Effects*

Typically, 300 increments are collected in $t_1$, each with 48 or 64 scans and 1024 complex data points collected in $t_2$. Our NMR data were processed using the program vnmr (version 6.1C, Varian Inc., Palo Alto CA) and analyzed using the program FELIX (version 97, Accelrys, San Diego, CA).

The time required for performing a trNOESY or NOESY experiment is dependent on the concentration of the ligands and the mixing time. For example, at concentrations of approximately 0.3 mM, 20 hrs are required for both the ligand alone (900 ms mixing time, 48 scans) and for the ligand in the presence of TetC (300 mixing time, 64 scans). At ligand concentrations of 1 mM, the time required can be reduced by half. Collecting data on mixtures of ligands instead of single ligands further decreases the total time required for the trNOESY experiment.

In our studies, we arbitrarily chose to carry out the trNOESY experiments at either 20 $^{o}$C or 30 $^{o}$C, empirically discovering that the data improved slightly at the higher temperature for most ligands. Changing the temperature can alter the rate of exchange between ligand and TetC to thereby increase the possibility for detection of trNOEs in some cases where binding was not observed. Lower temperatures slow the exchange rate, while higher temperatures will increase the exchange rate. Thus, one way to address the important limitation of the trNOESY competition assay, which is that binding can only be detected for those compounds that have mM to μM dissociation constants, is to change the temperature and consequently the rate of exchange (*see* **Note 5**).

### *3.3 Sample Preparation*

The methods used to (1) identify of the optimal set of ligands to screen, (2) prepare the protein and ligand stock solutions, and (3) prepare of the protein/ligand mixtures, are described below in Subheading 3.1.1-3.1.3.

#### *3.3.1. Identification of the optimal set of ligands to screen for binding to TetC*

There are several strategies, including random screening of a suitable chemical database, which can be employed to identify sets of potential binders. In this study, two crystal structures of TetC are available (pdb access codes 1AF9 and 1A8D [47, 48], (http://www.rcsb.org/pdb/)), so we employed a structure-based approach.

##### 3.3.1.1. Computational methods to identify suitable ligands

The first step involved using the crystal structure of the protein to identify binding sites and to carry out a virtual screen of the Available Chemical Directory (ACD). Two surface binding sites, Site-1 and Site-2, are identified by calculating the solvent

accessible surface and using the SPHGEN routine from DOCK 4.01 [49], which packs clusters of spheres into structural pockets. A sphere-atom matching scheme in the DOCK 4.01 program [50-52] is then used to computationally screen the ACD, which currently contains more than 300,000 commercially available compounds, and to predict which molecules will likely bind to Sites 1 and 2. A variety of structurally diverse ligands are chosen to represent the spectrum of possible candidates, and the best candidates are ranked by energy and contact scores. The top 1000 compounds are then visually examined qualitatively to assess the interactions they form with the site.

3.3.1.2. Further reduction and optimization of ligand set

The next step is to further scale down the top 100 compounds obtained from the virtual screen to approximately 10-20 compounds for each site, using a selection criteria based on cost and availability. The final cut is made by checking the remaining compounds for binding activity by electrospray ionization mass spectroscopy (ESI-MS) [53, 54]. Although this step is not required, it does help to significantly speed up the process in narrowing down the number of likely candidates. The sequential funneling of 300,000 ligands to about a dozen for each site resulted in optimizing the short list of compounds (Figure 4) to use in our NMR screening studies for binding activity in an aqueous environment. There are several reasons why checking for binding activity in solution is important. In particular, it is a prerequisite for identifying detection reagents that will be used in an aqueous environment. In addition, the synthesis of novel bidendate ligands requires that the individual compounds are compatible with one another and with TetC under similar solvent conditions.

*3.3.2 Preparation of stock solutions and sample solutions.*

1. Store dry recombinant TetC at –15 to –25 $^{o}$C (*see* **Note 6**).
2. Dissolve 1-3 mg dry (lyophilized) TetC protein samples in 500 µl / experiment of 100% $D_2O$ to obtain a final concentration between 38 and 72 µM. (*see* **Note 7**).
3. Centrifuge at highest speed for 5 minutes to remove any insoluble material before adding ligands.
4. Prepare concentrated stock solutions of ligands, either in 100 µl $D_2O$ or deuterated DMSO. The goal is to keep the total concentration of DMSO at or below 5% (v/v). The addition of this small amount of DMSO would not be expected to affect the stability of the protein, as previously demonstrated by other NMR studies [16], but it should be checked experimentally for each protein by determining if the binding activity is still detectable for a known binder when the protein is in a 5% DMSO solution. Three ligands, MP-biocytin, lavendustin A, and NF-galactopyranoside, were dissolved in deuterated DMSO; the remaining ligands (Figure 4) were all soluble in $D_2O$.
5. Calculate the volume of ligand stock solution required to prepare approximately 0.3 to 1 mM concentration in the final 500-600 µL sample volume. Test different molar ratios of TetC:doxorubicin ranging from 1:5 to 1:50 in order to determine the best ratios for use in the experiments. (*see* **Note 8**).
6. Prepare protein/ligand complexes by one of two methods. (1) Dissolve 3 mg of TetC in 1.0 ml of $D_2O$ (57.9 µM) and centrifuged for 5 minutes in an

Eppendorf microcentrifuge at highest speed to remove insoluble material prior to adding the ligands. (2) Prepare mixtures of ligands in ~ 500 to 800 µl of $D_2O$ (0.2 to 1 mM) prior to the addition of 1 to 3 mg of dry TetC (38 to 72 µM) to a final ratio of TetC:ligand of approximately 1:20.

### *3.4 The Screening/Competition trNOESY Assay*

Three screening/competition methods are described (1) sequential addition of ligands to TetC, (2) evaluation of ligands binding to TetC in reverse order, and (3) addition of TetC to different combinations of ligands for high throughput analysis.

The trNOESY competition assay requires a 'marker' ligand, for which the binding site on the protein and preferably the binding constant are known. The crystal structures of BoNT/B ligand complexes show that doxorubicin [55] and 3'-sialyllactose [56] bind to Site-1 (Figure 5). Site-1 is a common surface feature found in the structures of both TetC and BoNT, and preliminary results obtained from the crystal structure of TetC in complex with doxorubicin indicate that doxorubicin also binds to Site-1 on TetC (S. Swaminathan, personal communication). Furthermore, the dissociation constant for the doxorubicin/TetC complex is known and is approximately 10 µM [57]. Doxorubicin is thus an ideal marker ligand to use in our study.

The competition experiments are used to identify those compounds that bind to Site-1 or a different site by determining if binding of doxorubicin to TetC is disrupted by a competing ligand binding to the same site. This is evidenced by an absence of trNOEs for doxorubicin and the presence of trNOEs for the other ligand. However, it must be

stressed that these assays cannot identify the exact site of ligand binding, only if the sites are different or the same (*see* **Note 9** for additional caveats).

*3.4.1 Method 1: Sequential Addition of Ligands*.

Sequential addition of each of the computationally predicted Site-2 ligands is used as a negative control to show whether any of these ligands will also bind to Site-1 by displacement of doxorubicin. The results also show whether two or more ligands can bind simultaneously to TetC, thus identifying suitable pairs of ligands to link together in developing the synthetic high affinity ligands (SHALs) as detection reagents for the *Clostridium* neurotoxins.

1. Prepare a TetC:doxorubicin complex with the concentrations ~ [50 μM]/[1000 μM] or ratio of 1:20. Collect trNOESY data
2. Add ~ 1 mM of each of the predicted Site-2 ligand sequentially (Table 2), collecting trNOESY data after each addition.
3. Analyze trNOESY data after each addition to determine whether crosspeaks belonging to doxorubicin or added ligand become weaker, positive or disappear indicating that doxorubicin has been displaced. The appearance of strong negative crosspeaks indicate that the ligand is able to bind simultaneously with doxorubicin and is thus a good candidate for linking to another ligand (Figure 6).

*3.4.2. Method 2: Evaluation of ligands binding sequentially in reverse order to TetC.*

It is important to carry out the positive control, or the reverse experiment of Method 1. That is each ligand shown in Figure 4 is first added to TetC in 20 fold access to determine if binding could be observed without the interference possible due to the presence of another ligand. Doxorubicin is added next to the mixture to determine if it can displace the bound ligand being tested, and to confirm that the protein is active if no binding had occurred with ligand-1. If displacement occurs, than ligand-1 is identified as a Site-1 binder that exhibits a weaker binding affinity than the marker ligand, doxorubicin. Next, another ligand, ligand-3, is added to determine if the ligand-1 or doxorubicin is displaced. If ligand-1 is displaced but not doxorubicin, then ligand-1 and 3 must compete for the same site, with the ligand-3 having the higher binding affinity. A comprehensive analysis of the optimized short list of compounds using this strategy yielded the results shown in Figure 7. A step-by-step example of the method is described below.

1. Add 20 fold excess of sialic acid to TetC (Table 2) and acquire a 300 ms trNOESY spectrum (Figure 8A). Sialic Acid does not appear to bind.

2. Add 20 fold excess doxorubicin to the sialic acid/TetC solution. Acquire a 300 ms trNOESY spectrum (Figure 8B) to confirm that the protein has not lost its activity or that the batch of protein used was bad.

3. Add 20 fold excess of 3'-sialyllactose (another Site-1 binder) to the sialic acid/TetC/doxorubicin solution. Acquire a 300 ms trNOESY spectrum. In this case, 3'-sialyllactose displaced doxorubicin, confirming that both ligands bind to Site-1, with 3'-sialyllactose having a higher binding affinity constant than doxorubicin.

*3.4.3. Method 3: Addition of TetC to Different Combinations of Ligands*.

This method screens multiple ligands simultaneously for binding to TetC in a more high throughput manner. Up to ten ligands at a time can be rapidly screened, but since the set of ligands had been prescreened by computational and mass spectroscopy methods, we limited our combinations to three at a time, as shown in Figure 8. The time lost by running a larger number of trNOESY experiments is easily gained back by having to analyze much less complicated, and unambiguous, data sets. The experimental results also provided key information about the compatibility of the ligands under the same solvent conditions.

## 4. NOTES

1. Negative NOE crosspeaks for small molecules in the absence of TetC were sometimes observed when the molecule contains protons attached to large ring systems, such as the protons attached to the four-ring system of doxorubicin. These protons exhibit less internal motion than those located in more flexible long carbon chains. However, although the sign of the trNOE crosspeaks corresponding to these aromatic resonances remained the same regardless of whether TetC was present or not, their intensities were always much stronger for the bound ligand. Thus, both the sign and intensity of the crosspeaks must be taken into consideration when distinguishing ligands that bind from those that do not.
2. Our experiments were carried out on a Varian Inova 600 MHz spectrometer. Although the experiments can be carried out on a lower field instrument, higher

concentrations of both protein and ligands will be required because of the sensitivity of the experiment decreases with decreasing magnetic field strength.

3. This step is only necessary if the protein concentration is high and/or the molecular weight of the protein is low. Either case would result in the detection of protein signals that may interfere with the observation of the ligand signals. In our case, the TetC concentration is kept low (~ 50 μM). Although TetC signals could be detected at higher concentrations (~ 72 μM), they did not interfere with the detection of the ligand signals. In addition, the molecular weight of TetC is sufficiently large (51 kDa) and it is known to dimerize, so that the protein signals are broadened out into the baseline.

4. If the ligands and proteins can be dissolved in deuterated solvents and buffers, then a simple presaturation pulse of the water signal can suffice to eliminate any residual water signal. However, if the samples need to be prepared in solvents or buffers that contain a high ratio of $H_2O/D_2O$, then it is necessary to implement one of these water suppression schemes.

5. Sialic acid did not bind TetC at 2, 10, 20, 30, or 37 $^oC$, as evidenced by the presence of weak positive crosspeaks in the spectra at all of these temperatures. However, a crystal structure of a TetC/sialic acid complex by Emsley *et al.* [58] shows that sialic acid binds to a site that is adjacent to Site-1. One possible explanation for the discrepancies between the NMR and x-ray results is that sialic acid binds TetC with $< 10^3$ M or $> 10^6$ M affinity in the temperature range we tested. Another reason, however, may be due to differences in binding between the crystal state and the solution state.

6. While TetC can be purchased as a dry, lyophilized powder, other proteins may not be available dried or they cannot be dried without comprising their activity or risking their precipitation. In these cases, either the protein is dialyzed against deuterated buffer or the experiment is carried out in 90% $H_2O$/10% $D_2O$.

7. The minimum volume needed for the NMR experiment in a standard 5 mm tube is approximately 500 µl. However, other NMR tubes and probes could be used instead to reduce the amount of sample needed in cases were the protein is rare or has limited solubility. For example, 5 mm Shigemi tubes (200-300 µL) or a microprobe (2 µL) can be used to reduce the volumes. However, it should be noted that these are more expensive options and not always available in every NMR lab.

8. Ligand:Protein ratios between 5 and 50 can be used depending on the amount of protein available. The optimal molar ratio of TetC:doxorubicin was found to be between 1:15 and 1:25 and these ratios provided good sensitivity for detection of trNOEs for a large range of structurally unrelated ligands

9. Several limitations of trNOESY competition assays need to be considered: The possibility that two or more ligands can bind the same site simultaneously can not be completely ruled out based only on their lack of competition. Recently, Ma et al [59] have proposed that a protein can preexist in ensembles of sub-states, as a result of its conformational flexibility, and present a range of different binding site shapes to the incoming ligands, such that one site may recognize and bind multiple diverse ligands. An assumption has been made that the ligands are binding specifically to one site only, which may not always be the case. For example, N-acetylgalactosamine binds to two different sites in the crystal structure of the TetC complex [58]. Similarly, if a

predicted Site-2 ligand is binding with specificity to more than one site, a stronger competitor can displace it from one site, but not necessarily from the other sites. In such a case, both ligands would bind simultaneously to TetC. Non-specific association of a ligand with TetC is also possible, especially at very high concentrations of ligand.

## 5. Acknowledgements.

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***Table 1.*** **Ligands tested positive by ESI-MS for noncovalent complex formation with TetC**

| **Predicted Site-1** | **Predicted Site-2** |
|---|---|
| Doxorubicin[1,2] | Tyr-Glu-Trp |
| 3'-Sialyllactose[2] | Lavendustin A |
| D-(+)-cellotetraose[1] | Sar-Arg-Gly-Asp-Ser-Pro |
| Neohesperidin diHCl[1] | Naphthofluorescein di-(β-D-galactopyranoside) |
| Gly-Arg-Gly-Asp-Ser[1] | 3-(N-maleimidopropionyl) biocytin |
| Hemorphin-5[1] | Ser-Gln-Asn-Tyr-Pro-Ile-Val |
| Etoposide phosphate | |

[1]Reported in [57]. [2]Binds Site-1 in BoNT/B [55]

***Table 2.*** **Concentrations of Ligands and TetC and Observation of Binding Activity**.

| Figure | Ligand | [Lig] (mM) | [TetC] (mM) | [TetC]:[Lig] | binding |
|---|---|---|---|---|---|
| **Figure 6** | | | | | |
| A | Doxorubicin | 1081.4 | 54.3 | 1:20 | yes |
| B | Sar-RGDSP | 1116.3 | 50.6 | 1:22 | no |
| C | SQNYPIV | 1050.6 | 47.4 | 1:22 | yes |
| D | Lavendustin A | 993.3 | 45.0 | 1:22 | yes |
| E | NF-GalPyr | 926.6 | 43.3 | 1:21 | maybe[1] |
| **Figure 8** | | | | | |
| A | Sialic acid | 386.7 | 22.3 | 1:17 | no |
| BC | Doxorubicin | 365.6 | 21.5 | 1:17 | yes,no[2] |
| C | 3'-Sialyllactose | 351.9 | 20.7 | 1:17 | yes |

[1]Precipitation was observed, resulting in a decrease in the intensities of all crosspeaks. [2]Doxorubicin binding was observed in 8B but not in 8C.

## Figures

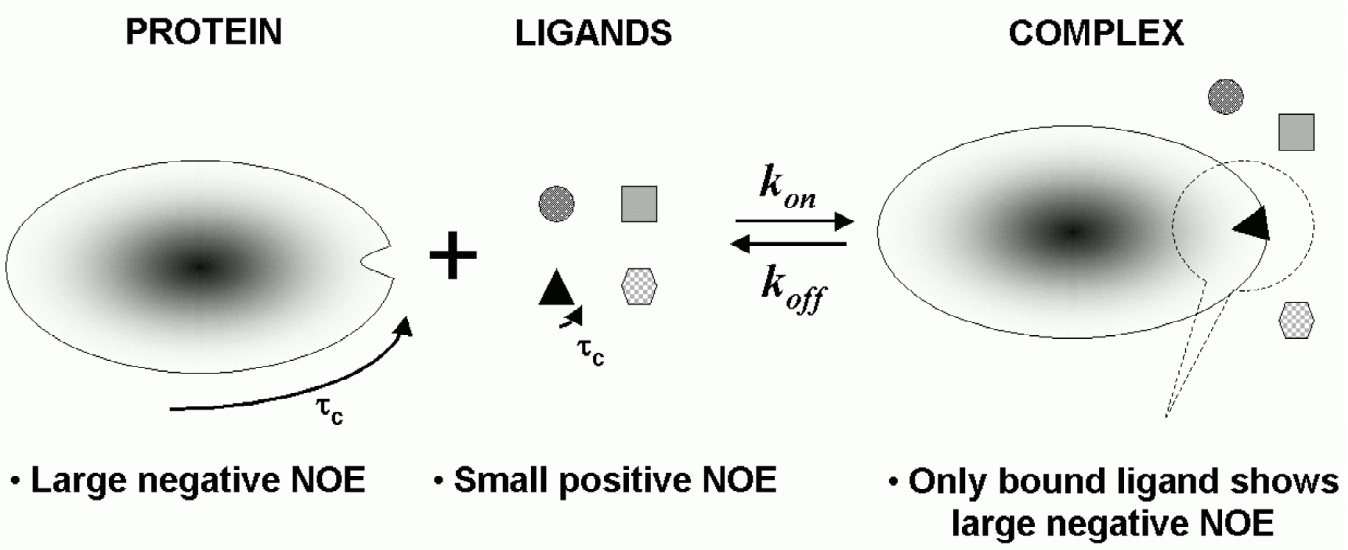


**Figure 1:** Principle of the experiment. The trNOE is only operative when the ligand is bound, since the ligand experiences a long correlation time ($\tau_c$) only in the bound state. The NOE is transferred and measured on the resonances of the ligand in the free state.

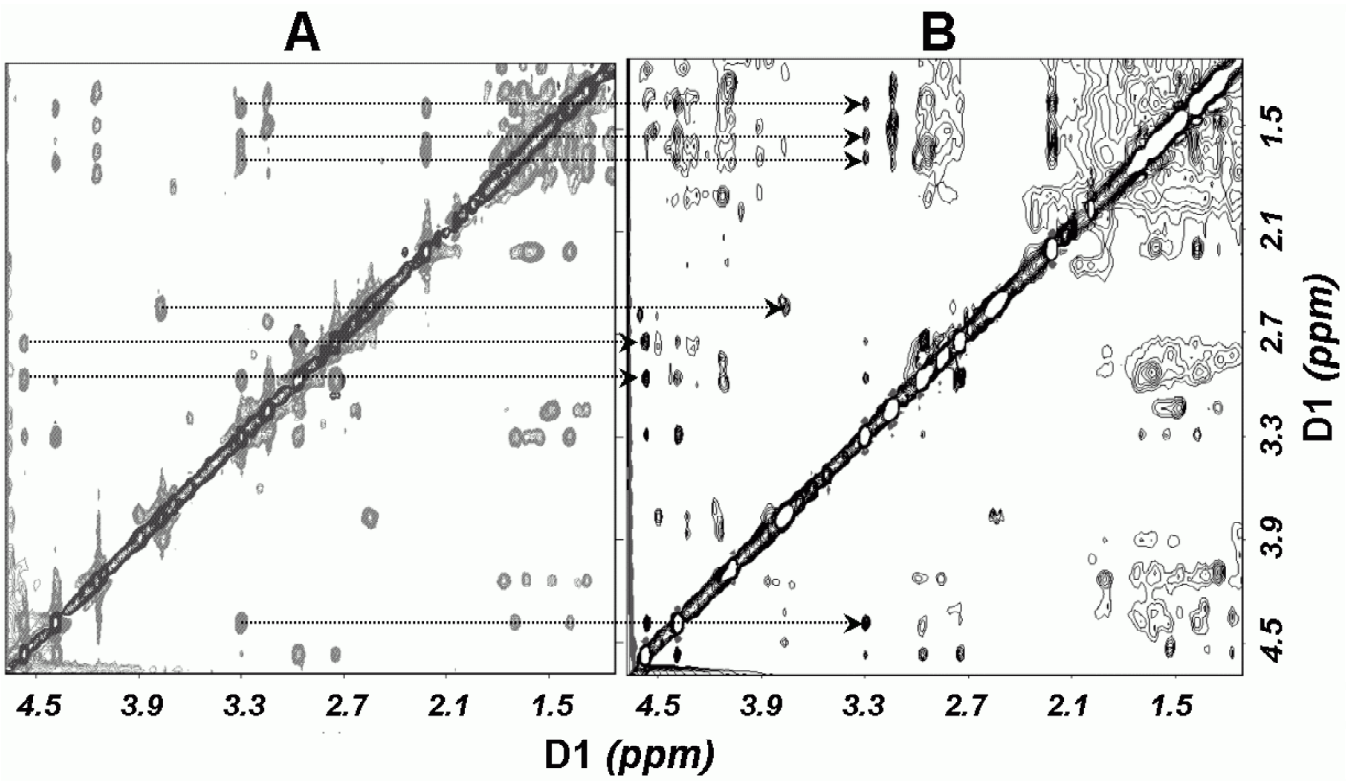


**Figure 2.** The trNOESY experiment: (A) 900 ms NOESY spectrum of MP-biocytin (~ 1 mM) exhibits weak positive crosspeaks (in gray). (B) Addition of TetC (~ 0.05 mM) results in the MP-biocytin crosspeaks in the 300 ms trNOESY to flip their sign to negative (in black) and increase in intensity, indicating that MP-biocytin binds to TetC. Several of the crosspeaks in both spectra are designated by the dashed arrows and the 900 ms spectrum in A is plotted at 2 times lower level than the 300 ms trNOESY spectra in B for presentation purposes. Spectra were acquired at 30 ◦C.

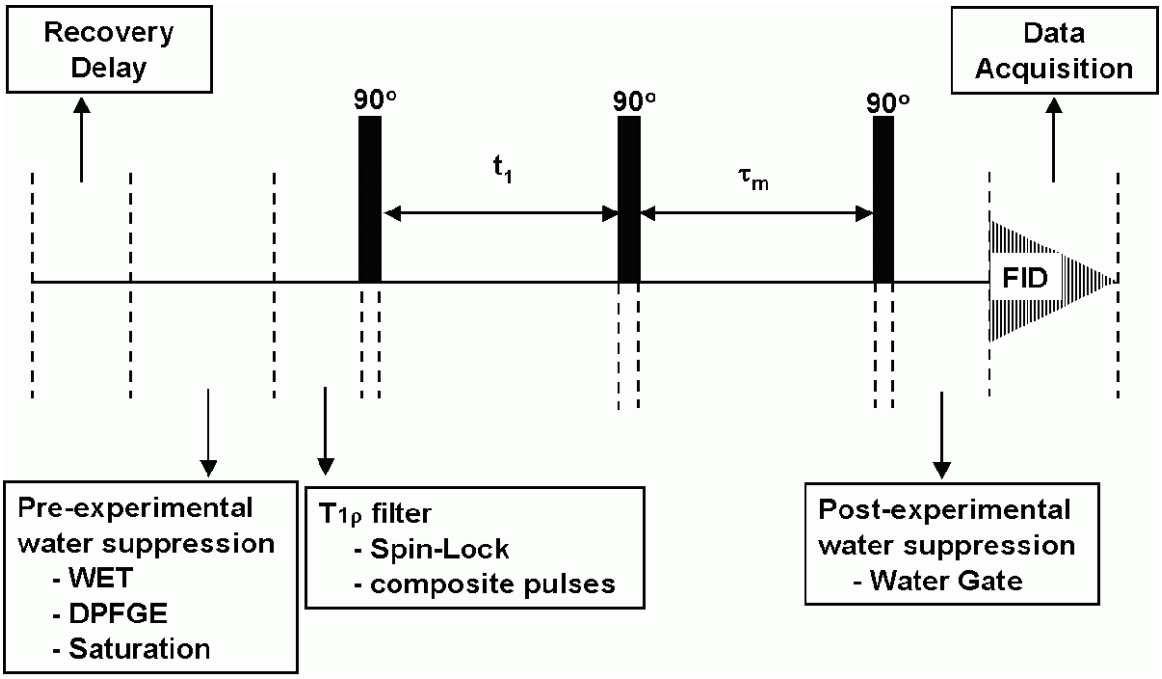


**Figure 3.** The trNOESY pulse sequence. Bars represent 90° radio-frequency pulses and FID stands for free induction decay. The pulses and the receiver are phase cycled to select the NOE and perform phase-sensitive detection along $t_1$.

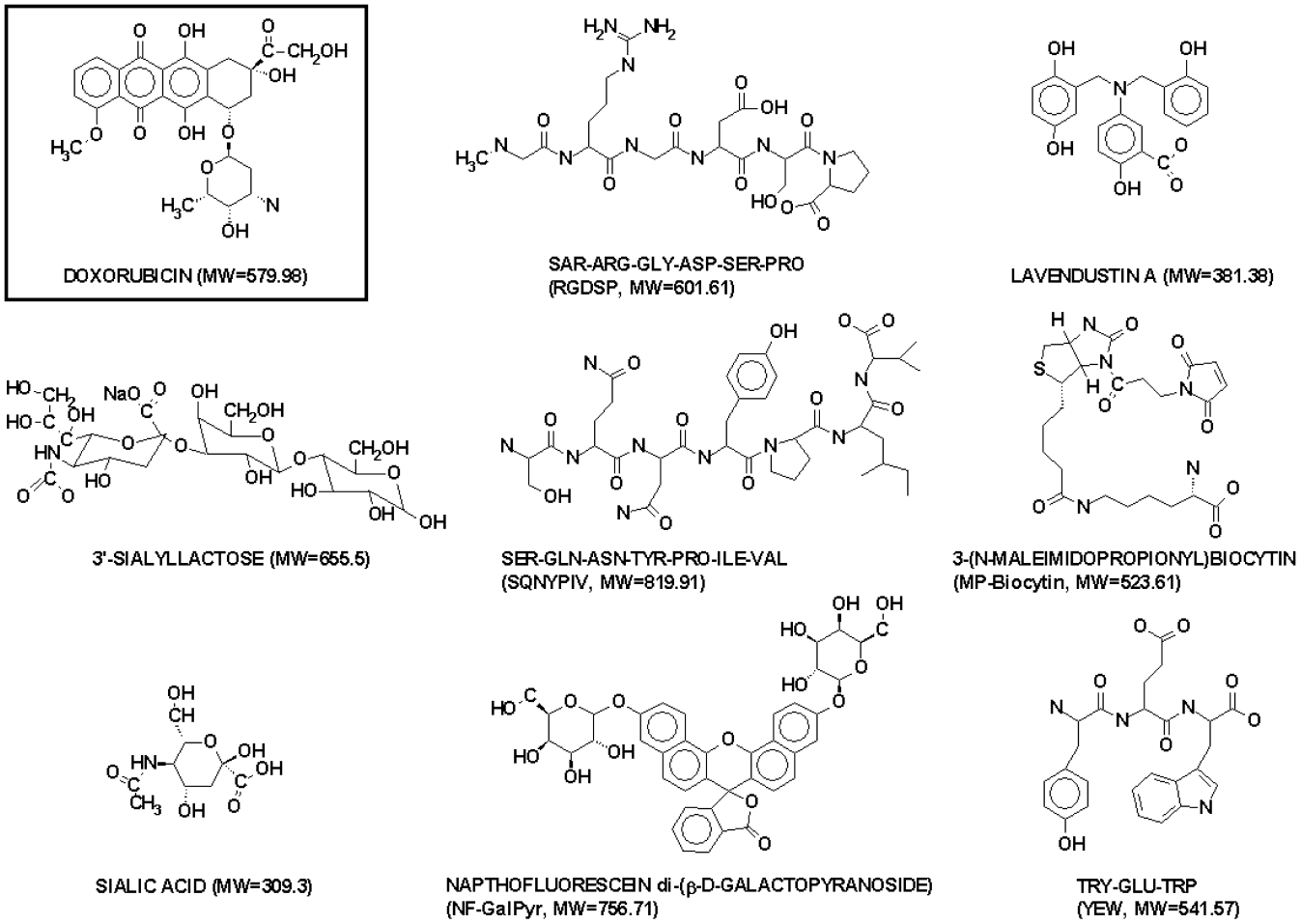


**Figure 4.** The structures and molecular weights of the ligands used in this study. The 'marker' ligand, doxorubicin, is boxed.

**Figure 5.** Crystal structure of TetC with doxorubicin, the marker ligand, computationally docked into Site-1. The figure was made using Molscript [60] and Raster3D [61] programs.

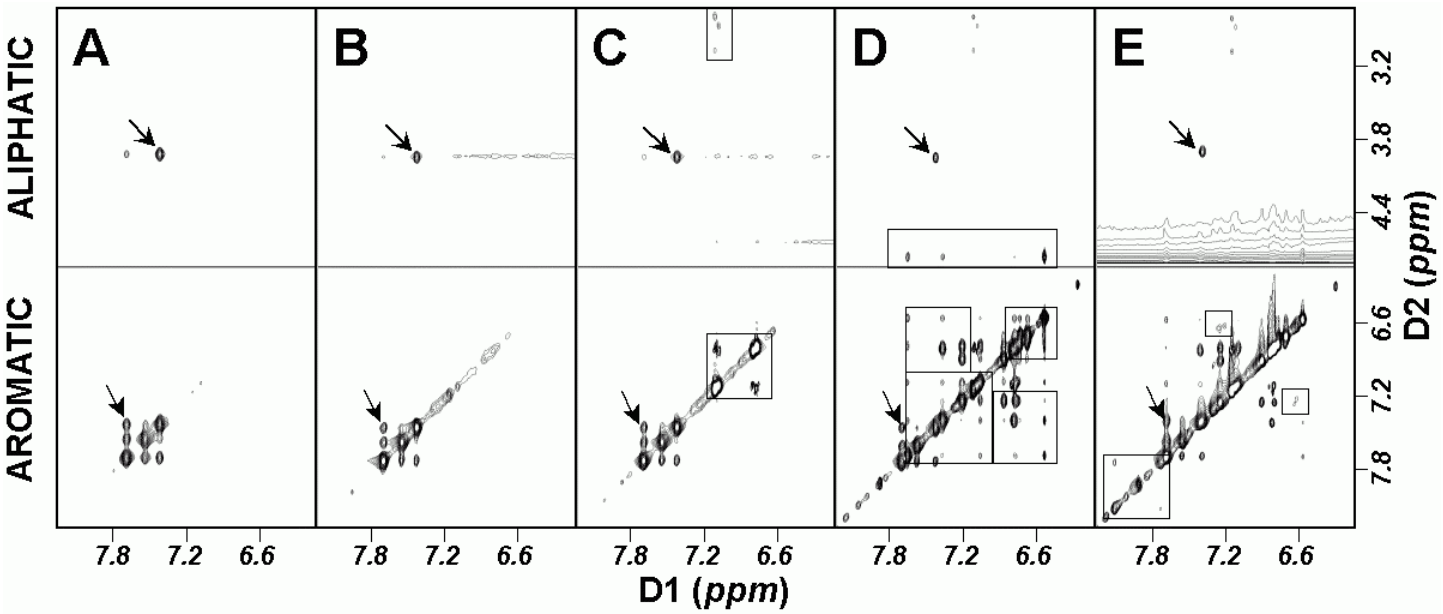


**Figure 6.** Effect of doxorubicin binding in Site-1 on predicted Site-2 ligands binding to TetC: Expanded regions of the 2D trNOESY spectra of TetC/ligand complexes at 200 ms mixing time and 20 °C showing binding of (A) doxorubicin (Site-1 marker ligand). The arrows indicate one aliphatic and one aromatic crosspeak that belongs to doxorubicin, which remains bound throughout the sequential additions of (B) Sar-RGDSP, (C) SQNYPIV, (D) Lavendustin A, and (E) NF-GalPyr. The appearance of new, negative crosspeaks in each panel are boxed, and indicate that Sar-RGRSP does not bind when doxorubicin is present but both SQNYPIV and lavendustin A do. The addition of NF-GalPyr (panel E), appears to partially displace lavendustin A, but not doxorubicin and SQNYPIV.

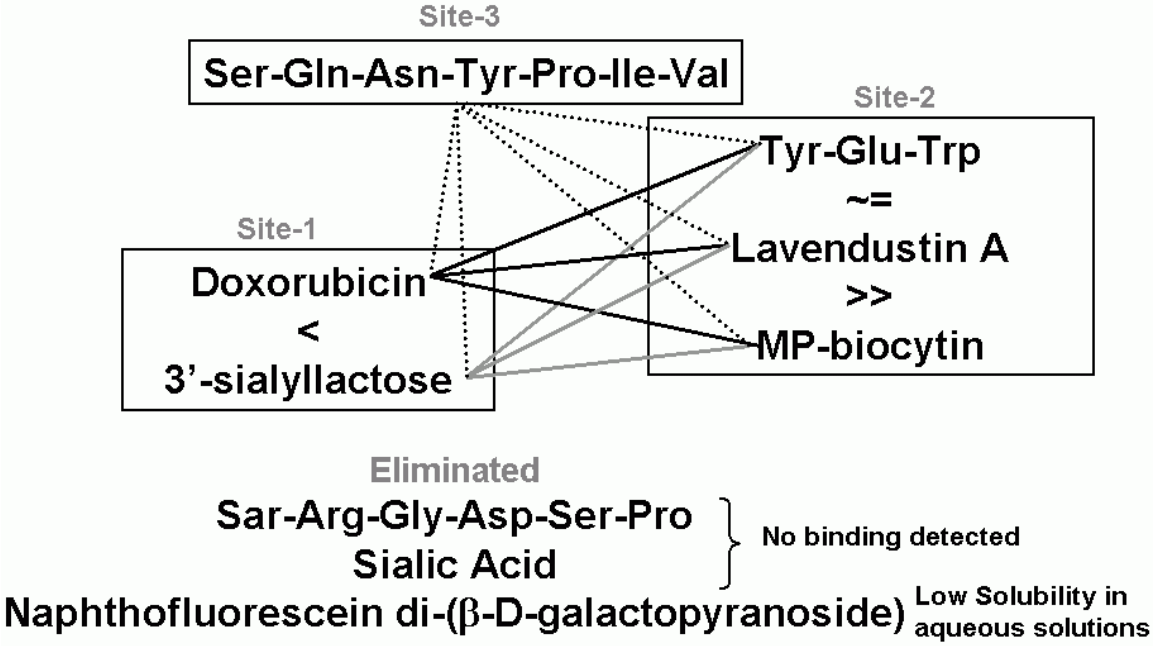


**Figure 7.** Grouping of optimized set of ligands tested by the trNOESY competition assay into three different binding sites on TetC. The lines indicate how a ligand from each site can be linked in unique ways to develop SHALs as detection reagents in nanosensors. Three ligands were eliminated because either binding was not detected or the ligand was insoluble in aqueous solutions.

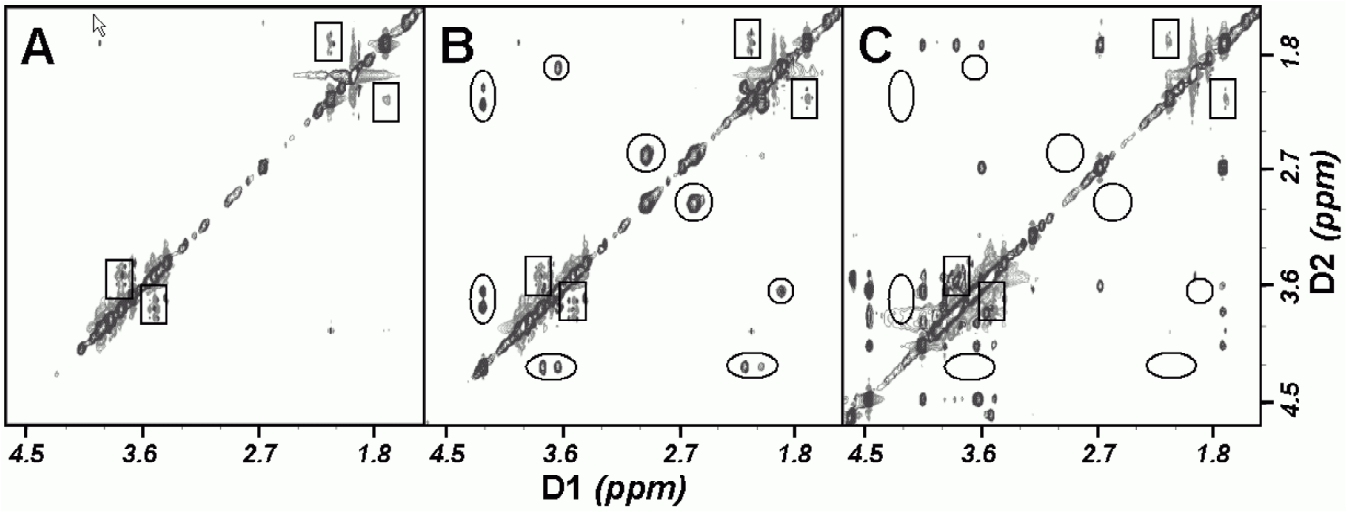


**Figure 8.** 300 ms trNOESY spectra at 2 ◦C of (A) sialic acid, which does not appear to bind to TetC, as evidenced by weak and positive trNOEs (gray boxed crosspeaks); (B) the addition of doxorubicin to a mixture of TetC and sialic acid, which results in doxorubicin binding as evidenced by the strong negative trNOEs, (black circled crosspeaks); and (C) the addition of 3'-sialyllactose to the mixture of sialic acid, doxorubicin and TetC shows that 3'-sialyllactose displaces doxorubicin from binding in Site-1. The circled positions of doxorubicin crosspeaks from (B) and the boxed gray sialic acid crosspeaks are shown in (C) for comparison.